EWS-FLI1 Fusion Protein Up-regulates Critical Genes in Neural Crest Development and Is Responsible for the Observed Phenotype of Ewing’s Family of Tumors

Siwen Hu-Lieskovan, Jingsong Zhang, Lingtao Wu, Hiroyuki Shimada, Deborah E. Schofield, and Timothy J. Triche

Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, California

Abstract

Tumor-specific translocations are common in tumors of mesenchymal origin. Whether the translocation determines the phenotype, or vice versa, is debatable. Ewing's family tumors (EFT) are consistently associated with an EWS-FLI1 translocation and a primitive neural phenotype. Histogenesis and classification are therefore uncertain. To test whether EWS-FLI1 fusion gene expression is responsible for the primitive neuroectodermal phenotype of EFT, we established a tetracycline-inducible EWS-FLI1 expression system in a rhabdomyosarcoma cell line RD. Cell morphology changed after EWS-FLI1 expression, resembling cultured EFT cells. Xenografts showed typical EFT features, distinct from tumors formed by parental RD. Neuron-specific microtubule gene MAPT, parasympathetic marker cholecystokinin, and epithelial marker keratin 18 were up-regulated. Conversely, myogenesis was diminished. Comparison of the up-regulated genes in RD-EF with the Ewing's signature genes identified important EWS-FLI1 downstream genes, many involved in neural crest differentiation. These results were validated by real-time reverse transcription-PCR analysis and RNA interference analysis using small interfering RNA against EWS-FLI1 breakpoint. The present study shows that the neural phenotype of Ewing's tumors is attributable to the EWS-FLI1 expression and the resultant phenotype resembles developing neural crest. Such tumors have a limited neural phenotype regardless of tissue of origin. These findings challenge traditional views of histogenesis and tumor origin. (Cancer Res 2005; 65(11): 4633-44)

Introduction

Mesenchymal tumors often harbor characteristic chromosome translocations (1). The consistency and tumor specificity of these translocations imply a close relation between the fusion proteins as a result of the translocations and certain tumor phenotypes. One possible explanation is that a given translocation can only occur in a certain determined cell lineage where the right cellular background exists to tolerate and cooperate with the fusion protein. Alternatively, distinct fusions may occur in common multipotent undifferentiated precursor cells and influence cell development, subsequently driving the cells towards different phenotypes or lineages. A good model to investigate these possibilities is the Ewing's family tumors (EFT), a group of poorly differentiated pediatric and young adult cancers of bone and soft tissue, which harbors characteristic translocations in virtually all cases. These translocations fuse a heretofore unknown gene, termed EWS, on chromosome 22, with a member of the ETS family of developmentally regulated genes, most commonly FLI1 on chromosome 11 (2). These fusion proteins function as aberrant transcription factors. Numerous investigations have now documented the near universal association of one of these translocations with the tumor.

The occurrence of EWS-FLI1 (EWS-ETS) translocation(s) has enabled grouping of a spectrum of seemingly unrelated tumors with various degrees of neuroectodermal differentiation into one family: from typical undifferentiated Ewing's sarcoma to poorly differentiated atypical Ewing's sarcoma to differentiated peripheral primitive neuroectodermal tumor (pPNET). The cell lineage that EFT originates from is still somewhat enigmatic. However, a parasympathetic neural crest origin has been suggested because some of the tumors express limited degree of neural markers (e.g., cholecystokinin [CCK]; ref. 3) and they can be induced to undergo neural differentiation by various differentiating agents (4, 5). EWS-FLI1 has been considered a traditional "oncogene" (i.e., promoting the proliferation and blocking the differentiation of a committed neural crest precursor cell). Indeed, early experiments that down-regulated expression of the chimeric gene resulted in diminished proliferation (6). However, it was later discovered that simple transfection of the EWS-FLI1 gene was generally lethal and certainly did not accelerate cell proliferation (7, 8). This in fact has been true of most such chimeric oncogenes when simply transfected into normal or tumor cell backgrounds and indicates that secondary genetic alterations are required for EWS-FLI1-mediated transformation.

In contrast to its role in oncogenesis, EWS-FLI1 seems to inhibit tissue-specific differentiation. Forced EWS-FLI1 expression inhibited osteogenic and adipogenic differentiation in marrow stromal cells (9), myogenic differentiation in C2C12 cells (10), and sympathetic neural differentiation in neuroblastoma cells (11). Interestingly, tumors formed by EWS-FLI1--transformed NIH3T3 cells, an immortalized murine fibroblast line, acquired a certain degree of neural features and a small round cell morphology, which is typical of EFT but distinct from fibrosarcomas (12). This suggests a possible role of EWS-FLI1 in inhibiting tissue-associated differentiation but promoting an Ewing-specific neuroectodermal differentiation program in these tumors. Further evidence is a group of biphenotypic soft tissue sarcomas. They contain the same EWS-FLI1 or EWS-ERG fusions and manifest a lesser degree of myogenic differentiation than rhabdomyosarcoma with no translocations whereas displaying some neural features (13–15).

In this study, we established a tetracycline-regulated EWS-FLI1 expression model in RD, an embryonal rhabdomyosarcoma cell line...
with marked myogenic differentiation, to test the hypothesis that EWS-FLI1 fusion protein is responsible for the observed primitive neuroectodermal phenotype of EFT, by the regulation of genes involved in cell proliferation and differentiation.

**Materials and Methods**

**Transfection and selection of clones.** EWS-FLI1 type I fusion cDNA was cloned into pcDNA4/TO (Invitrogen, San Diego, CA), a tetracycline-inducible expression vector, and named pcDNA4/TO-EF. The construct was verified by DNA sequencing. Effectene (Qiagen, Chatsworth, CA) was used for all transfections following manufacture's suggestions. To establish the tetracycline-regulated system (TREx, Invitrogen), RD cells were first stably transfected with pcDNA6/TR and monoclones were selected and maintained in tetracycline-free medium containing 5 μg/mL Blasticidin (Invitrogen). The inducibility of each clone was tested by transient transfection with pcDNA4/TO/LacZ and stained for β-gal (Invitrogen). Two of 40 pcDNA6/TR clones were chosen for second stable transfection with pcDNA4/TO-EF. Tetracycline-free medium containing 5 μg/mL Blasticidin and 400 μg/mL Zeocin (Invitrogen) was used for selecting and maintaining monoclones. Induction of EWS-FLI1 was accomplished by adding 1 μg/mL tetracycline to media and tested by reverse transcription-PCR (RT-PCR) and Western blot.

**Tumors and cell lines.** Frozen primary tumor tissues were obtained before therapy from Children's Hospital Los Angeles (CHLA). All cell lines were obtained from our cell bank and cultured in RPMI with 10% fetal bovine serum (Invitrogen). Fusion gene status was tested by RT-PCR. Among the 16 EFT, 36 rhabdomyosarcoma, 10 neuroblastoma, and 20 osteosarcoma samples used for gene expression analysis, EFT samples were chosen for second stable transfection with pcDNA4/TO-EF. RD-EF leaky vector, and named pcDNA4/TO-EF. The construct was verified by DNA sequencing. Effectene (Qiagen, Chatsworth, CA) was used for all transfections following manufacture's suggestions. To establish the tetracycline-regulated system (TREx, Invitrogen), RD cells were first stably transfected with pcDNA6/TR and monoclones were selected and maintained in tetracycline-free medium containing 5 μg/mL Blasticidin (Invitrogen). The inducibility of each clone was tested by transient transfection with pcDNA4/TO/LacZ and stained for β-gal (Invitrogen). Two of 40 pcDNA6/TR clones were chosen for second stable transfection with pcDNA4/TO-EF. Tetracycline-free medium containing 5 μg/mL Blasticidin and 400 μg/mL Zeocin (Invitrogen) was used for selecting and maintaining monoclones. Induction of EWS-FLI1 was accomplished by adding 1 μg/mL tetracycline to media and tested by reverse transcription-PCR (RT-PCR) and Western blot.

**Microarray analysis.** For tumor samples, tissues that had >90% tumor cell counts were chosen. For cultured cell lines, RNA was harvested when cell confluence reached 50% to 60%. Total RNA were extracted (RNA STAT-60, Tel-Test, Cleared (RNeasy mini kit, Qiagen), and quantitated. Synthesis of cDNA, biotin-labeled cRNA, fragmentation, target hybridization, washing, staining, and scanning probe arrays followed Affymetrix's manual at CHLA microarray core facility. The Affymetrix HU95av2 array was hybridized for 16 hours, washed, stained, and scanned with a GeneArray scanner. The microarray core facility at the University of California, San Diego, provided hybridization, scanning, and data analysis services. Bioinformatical analyses were done with Genetrix analysis software (Epicenter Software, Pasadena, CA).

**Real-time quantitative reverse transcription-PCR.** Total cellular RNA was isolated using RNA STAT-60 (Tel-Test) when cells reach 50% to 60% confluence. cDNA was synthesized from 2 μg of DNase I (Invitrogen)–treated total RNA in a 42°C incubation with biotinylated anti-mouse or anti-rabbit IgG (Invitrogen). Two of 40 pcDNA6/TR clones were chosen for second stable transfection with pcDNA4/TO-EF. Tetracycline-free medium containing 5 μg/mL Blasticidin and 400 μg/mL Zeocin (Invitrogen) was used for selecting and maintaining monoclones. Induction of EWS-FLI1 was accomplished by adding 1 μg/mL tetracycline to media and tested by reverse transcription-PCR (RT-PCR) and Western blot.

**Electron microscopy.** Electron microscopy (EM) was carried out by the standard procedure at the CHLA EM laboratory. Briefly, about 1-mm cubes from xenograft tumor samples were fixed with 2% glutaraldehyde in phosphate buffer (pH 7.4), post-fixed with 1% osmium tetroxide, and embedded in epon (Embed-812, Electron Microscopy Sciences, Hatfield, PA). One-micrometer-thick sections, cut from the hardened epon blocks and stained with 1% methylene blue and 0.5% basic fuchsin, were examined under the light microscope before ultrastructural examination. Ultrathin sections from the areas of interest were mounted on one-hole grids, stained with uranyl acetate/lead citrate, and examined and photographed with a Philips CM-12 transmission electron microscope.

**Immunohistochemistry.** Paraffin-embedded tissue sections were cut from 4-μm paraffin blocks and were treated with a microwave antigen retrieval protocol. Immunohistochemical staining was carried out using DAKO EnVision Detection System (DAKO, Carpinteria, CA). Antibodies were obtained from Dako (anti-desmin, anti-vimentin), M.O.M. Immunodetection Kit (Vector Laboratories, Burlingame, CA), BD PharMingen (anti-MyoD, anti-Myogenin, anti-MyoD1, anti-Myogenin), and Cell Signaling Technology (anti-Cyclin D1, anti-Cyclin D3, and anti-β-Actin). Staining was carried out according to the manufacturer's instructions. All sections were counterstained with hematoxylin before examination under the light microscope.
substrate (Sigma, St. Louis, MO) to give a brown stain and counterstained with Mayer’s hematoxylin. After washing with PBS and mounted, the sections were examined and photographed with a Nikon epifluorescent microscope.

**RNA interference.** Small interfering RNA (siRNA) against EWS-FLI1 breakpoint (siEFBP2) and a nontargeting control siRNA (C8) were obtained from Dharmacon Research (Lafayette, CO). Sequence of siEFBP2 was reported before (18). siEFBP2 or C8, complexed with TransIT-TKO (Mirus) in Opti-MEM I (Invitrogen) following manufacturer’s directions, were applied to RD-EF cells or TC32 cells 30% confluent in RPMI with 10% fetal bovine serum but without antibiotics in 6-well plates, to give a final concentration of 100 nmol/L. For RD-EF cells, 12 hours after transfection, the cells were induced by tetracycline or ethanol for another 24 hours before RNA was harvested. For TC32 cells, the same transfection was repeated after 24 hours and RNA was harvested after another 24 hours. Gene expression was assessed by QRT-PCR.

**Results**

**Establishment of the RD-EF model system.** We expressed the EWS-FLI1 fusion gene in RD cells by using the TREx system from Invitrogen, which allowed us to identify expression levels that are nonlethal but associated with profound effects on patterns of gene expression and cell differentiation. Several stable clones with high inducibility and low leakage were selected. Among them, B101 (RD-EF) was used for the subsequent experiments because tetracycline treatment led to strong induction of EWS-FLI1 in this clone. EWS-FLI1 RNA could be detected as early as 3 hours by QRT-PCR (Supplementary Fig. 1A). Western blotting analysis revealed a rapid induction of EWS-FLI1 proteins as early as 6 hours and reached the peak level at 36 hours (Fig. 1A). The level of EWS-FLI1 expression induced in the model system is comparable to that in wild-type EFT cell lines (Fig. 3B and D; Supplementary Fig. 1A). Fluorescent microscopy showed that induced EWS-FLI1 was localized to nucleus (data not shown). Ethanol (solvent of tetracycline) treatment of RD-EF cells did not induce EWS-FLI1 expression (Fig. 1A).

Because EWS-FLI1 functions as an aberrant transcription factor, to investigate the molecular consequences of EWS-FLI1 expression in RD cells, we undertook a transcriptome-wide gene expression analysis using Affymetrix HU95av2 gene chips, on samples harvested 0, 6, 12, 18, 24, and 36 hours after tetracycline or ethanol treatments (2-6 biological replicates at each time point, Fig. 1B). An additional set of RNA was harvested at 0, 6, 9, 18, 24, and 36 hours for confirmatory QRT-PCR using β actin as an internal control. We also compared the microarray data of the model system with a pool of data from 83 tumor samples, including 16 EFT, 36 rhabdomyosarcoma, 20 osteosarcoma, and 10 neuroblastoma (Fig. 1C).

**EWS-FLI1 expression in RD cells revealed a shift in global gene expression pattern with induction of EFT markers.** To provide visual representation of the samples based on gene

---

Figure 1. A global gene expression change and induction of EFT markers after EWS-FLI1 expression in RD cells.

**A,** Western blot analyses of RD-EF cells after tetracycline or ethanol treatments. The same blots probed with antibodies against FLI1 (top) or Actin (bottom).

**B,** schematic illustration of treatments at different time points for microarray analysis and the number of replicates at each time. E, ethanol treatments; T, tetracycline treatments.

**C,** PCA of induced RD-EF samples and tumor samples.

**D,** expression of known EFT markers and identified EWS-FLI1 targets in RD-EF samples and tumor samples by microarray analysis. Red line, tetracycline-treated samples; green line, ethanol-treated controls.
expression, we used principal component analysis (PCA; ref. 19) to locate the two-dimensional views that capture the greatest amount of variability in the data, using all 12,625 probe sets interrogating 10,500 genes. Continued expression of EWS-FLI1 chimeric gene induced a progressive shift away from both RD cells and ethanol-treated controls (Fig. 1C). All of the controls cluster together without an apparent pattern. However, tetracycline-treated samples cluster in a distinct pattern that correlates directly with the induced time. Longer exposure to tetracycline resulted in a greater accumulation of EWS-FLI1 protein, and data points separated progressively further from that of the controls. These data indicated a fundamental difference in gene expression pattern between the EWS-FLI1–expressing cells and the controls.

Detailed analysis of the differentially expressed genes revealed several noteworthy genes previously identified as EFT markers (CD99, ref. 20; CCK, ref. 3; and STEAP, ref. 21), or EWS-FLI1 downstream genes (c-MYC, ref. 22; ID2, refs. 23, 24; and Cyclin, ref. 25), all highly expressed in EFT, and also highly up-regulated in tetracycline-treated RD-EF cells (Figs. 1D and 2C, m-r). Interestingly, the up-regulation of c-MYC and ID2 (6 hours) preceded Cyclin D1 (18 hours), which implied that these two genes are early response genes and Cyclin D1 is possibly a secondary target of EWS-FLI1. EWS-FLI1 down-regulates transforming growth factor βRII (TGFβRII; ref. 26) and P21 (27). It seemed that ethanol itself could regulate the expression of TGFβRII. However, tetracycline-treated RD-EF cells showed more down-regulation than ethanol-treated cells. P21 expression

Figure 2. Cultured RD-EF with EWS-FLI1 expression and xenograft tumors formed by EWS-FLI1 expressing RD showed the phenotype of typical EFT. A, light microscopy revealed a consistent morphological change after EWS-FLI1 induction in RD cells. B, EM study showed that xenograft tumors formed by EWS-FLI1–expressing RD cells lost muscle differentiation features that normally can be found in tumors formed by wild-type RD cells, and acquired certain degree of neural features, such as neurites and dense core granules (dcg). C, H&E staining (a-c) and immunohistochemistry (d-r) of Desmin, muscle-specific actin (HHF35), MAPT, CCK, and CD99 on xenograft tumors formed by RD, EWS-FLI1–expressing RD, or TC32 cells.
decreased dramatically up to 18 hours. Thus, expression of EWS-FLI1 resulted in a marked shift in global gene expression, with induction and suppression of several known EFT markers and EWS-FLI1 target genes.

Cultured RD-EF cells and RD-EF xenograft tumors that express EWS-FLI1 acquired the phenotype of Ewing’s family tumors. We monitored RD-EF cell morphology preinduction and post-induction. Figure 2A illustrates the effect of only 24 hours of expression of EWS-FLI1 in this cellular context. The spindle-shaped RD (typical for cell lines from skeletal muscle lineage) gradually became polygonal with frequent cellular processes. By 24 hours, the morphology of the tetracycline-treated RD-EF cells resembled EFT cells (TC32) far more than untreated RD-EF or wild-type RD cells. Ethanol treatment did not show this effect. This data was in accordance with the observation in C2C12-EF cells (10), which exhibited a cuboidal appearance after EWS-FLI1 expression, and suggested that EWS-FLI1 expression mediated this morphologic change of RD.

The impression of probable neural induction observed by phase-contrast microscopy, was confirmed by electron microscopy and H&E staining of tumors derived from xenografts of RD cells, TC32 EFT cells, and RD cells expressing EWS-FLI1. RD cells normally form bona fide rhabdomyosarcoma tumors with marked terminal rhabdomyogenesis, whereas EFT cells are largely undifferentiated with scant neural differentiation marked by clusters of cytoplasmic dense core granules. Strikingly, RD cells that express EWS-FLI1 show near complete suppression of the myogenic phenotype with concomitant neural differentiation, marked by the appearance of neuritic processes containing dense core granules (Fig. 2B). H&E staining of RD-EF xenografts showed a structureless array of biphasic cell population (small dark and large light types) with scant cytoplasm (Fig. 2C, b), which is typical of EFT tumors (Fig. 2C, c). On the other hand, RD xenografts still showed typical rhabdomyosarcoma tumor phenotype (a diffuse infiltrate of small round-to-spindled cells in a collagenous stroma, Fig. 2C, a). Clearly, the EWS-FLI1 gene has a potent neural differentiating effect at the expense of the existing tissue differentiation. This dual effect has not been previously recognized.

Appearance of an Ewing-like neuroectodermal phenotype was accompanied by diminished myogenic differentiation in induced RD-EF cells. A genetic basis for these observations was explored by gene expression analysis. Two cytokeratin structural genes, microtubule associated protein tau (MAPT) and keratin 18, were up-regulated >8-fold after EWS-FLI1 expression (Fig. 3A; P < 0.001). MAPT is expressed exclusively in the axons of neurons and promotes microtubule assembly and stability. Both EFT and neuroblastoma but not rhabdomyosarcoma express MAPT at high levels. Keratin 18 is an epithelial structural protein. The up-regulation of these two genes implied that EWS-FLI1 induced a neuroectodermal phenotype in mesodermal RD cells, consistent with the morphologic results reported above. Western blot and immunohistochemical analysis showed that the MAPT protein started to accumulate at 24 hours and reached peak level at 48 hours (Figs. 2C, j-l and 3B), which suggested that MAPT takes part in the morphologic change induced by EWS-FLI1 expression.
Table 1. Genes (n = 109) significantly upregulated in RD-EF and also highly expressed in EFT

<table>
<thead>
<tr>
<th>Affy ID</th>
<th>Gene name</th>
<th>Symbol</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>33113_at</td>
<td>Chp/p300-interacting transactivator</td>
<td>CITED2</td>
<td>1.03e−12</td>
</tr>
<tr>
<td>37724_at</td>
<td>V-\textit{myc} myelocytomatosis viral oncogene homologue (avian)</td>
<td>MYC</td>
<td>3.28e−10</td>
</tr>
<tr>
<td>1973_s_at</td>
<td>Inhibitor of DNA binding 2, dominant-negative HLH protein</td>
<td>ID2</td>
<td>9.63e−10</td>
</tr>
<tr>
<td>41215_s_at</td>
<td>REST corepressor</td>
<td>RCOR</td>
<td>2.80e−09</td>
</tr>
<tr>
<td>37651_at</td>
<td>Hairy and enhancer of split 1, (\textit{Drosophila})</td>
<td>HESI</td>
<td>0.000000001</td>
</tr>
<tr>
<td>31862_at</td>
<td>Wingless-type MMTV integration site family, member 5A</td>
<td>WNT5A</td>
<td>0.000000008</td>
</tr>
<tr>
<td>1669_at</td>
<td>Protein tyrosine phosphatase, nonreceptor type substrate 1</td>
<td>PTPNS1</td>
<td>0.000000031</td>
</tr>
<tr>
<td>1135_at</td>
<td>G protein–coupled receptor kinase 5</td>
<td>GBP5</td>
<td>0.00000074</td>
</tr>
<tr>
<td>41216_r_at</td>
<td>Sialyltransferase 1 (\textit{f}-galactoside alpha-2,6-sialyltransferase)</td>
<td>SIAT1</td>
<td>0.000008</td>
</tr>
<tr>
<td>1916_s_at</td>
<td>V-fos FBJ murine osteosarcoma viral oncogene homologue</td>
<td>FOS</td>
<td>7.33e−12</td>
</tr>
<tr>
<td>40049_at</td>
<td>Death-associated protein kinase 1</td>
<td>DAPK1</td>
<td>8.83e−10</td>
</tr>
<tr>
<td>36976_at</td>
<td>Cadherin 11, type 2, OB-cadherin (osteoblast)</td>
<td>CDH11</td>
<td>0.000000001</td>
</tr>
<tr>
<td>2087_s_at</td>
<td>START domain containing 13</td>
<td>STARD13</td>
<td>0.000000005</td>
</tr>
<tr>
<td>36119_at</td>
<td>Caveolin 1, caveolae protein, 22 kDa</td>
<td>CAV1</td>
<td>0.000000001</td>
</tr>
<tr>
<td>38546_at</td>
<td>Interleukin 1 receptor accessory protein</td>
<td>IL1RAP</td>
<td>0.000000001</td>
</tr>
<tr>
<td>1457_at</td>
<td>Janus kinase 1 (a protein tyrosine kinase)</td>
<td>JAK1</td>
<td>0.0000000064</td>
</tr>
<tr>
<td>41126_at</td>
<td>Solute carrier family 1, member 4</td>
<td>SLCL14</td>
<td>0.0000000068</td>
</tr>
<tr>
<td>36502_at</td>
<td>Putative lymphocyte G0-G1 switch gene</td>
<td>G0S2</td>
<td>0.0000064</td>
</tr>
<tr>
<td>36502_at</td>
<td>Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF)</td>
<td>PPTF1</td>
<td>0.00012</td>
</tr>
<tr>
<td>38326_at</td>
<td>Myosin X</td>
<td>MYO10</td>
<td>0.000061</td>
</tr>
<tr>
<td>339_at</td>
<td>Glycogenin 2</td>
<td>GYG2</td>
<td>0.0000025</td>
</tr>
<tr>
<td>40199_at</td>
<td>Msh homeo box homologue 1 (\textit{Drosophila})</td>
<td>MSX1</td>
<td>2.27e−10</td>
</tr>
<tr>
<td>215_g_at</td>
<td>Glycogenin 2</td>
<td>GYG2</td>
<td>2.49e−10</td>
</tr>
<tr>
<td>40199_at</td>
<td>Msh homeo box homologue 1 (\textit{Drosophila})</td>
<td>MSX1</td>
<td>2.55e−10</td>
</tr>
</tbody>
</table>

(Continued on the following page)
Table 1. Genes (n = 109) significantly upregulated in RD-EF and also highly expressed in EFT (Cont’d)

<table>
<thead>
<tr>
<th>Affy ID</th>
<th>Gene name</th>
<th>Symbol</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>37706_at</td>
<td>Golgi apparatus protein 1</td>
<td>GLG1</td>
<td>0.00000005</td>
</tr>
<tr>
<td>1251_g_at</td>
<td>RAP1, GTPase activating protein 1</td>
<td>RAP1GA1</td>
<td>0.00000007</td>
</tr>
<tr>
<td>1737_s_at</td>
<td>Insulin-like growth factor binding protein 4</td>
<td>IGFBP4</td>
<td>0.00000007</td>
</tr>
<tr>
<td>39781_at</td>
<td></td>
<td></td>
<td>3.60e–09</td>
</tr>
<tr>
<td>39428_at</td>
<td>Lymphocyte adaptor protein</td>
<td>LNK</td>
<td>0.00000021</td>
</tr>
<tr>
<td>39151_at</td>
<td>Loss of heterozygosity, 11, chromosomal region 2, gene A</td>
<td>LOH11CR2A</td>
<td>0.00000866</td>
</tr>
<tr>
<td>32598_at</td>
<td>NEL-like 2 (chicken)</td>
<td>NEL2</td>
<td>0.00000876</td>
</tr>
<tr>
<td>38418_at</td>
<td>Cyclin D1 (PRAD1: parathyroid adenomatosis 1)</td>
<td>CCND1</td>
<td>0.000011</td>
</tr>
<tr>
<td>40618_at</td>
<td></td>
<td></td>
<td>0.000016</td>
</tr>
<tr>
<td>38017_at</td>
<td>Adenylate kinase 3</td>
<td>AK3</td>
<td>0.00026</td>
</tr>
<tr>
<td>642_s_at</td>
<td>Presenilin 1 (Alzheimer disease 3)</td>
<td>PSEN1</td>
<td>0.00045</td>
</tr>
<tr>
<td>39376_at</td>
<td>Homedomain interacting protein kinase 1-like protein</td>
<td>Nbak2</td>
<td>0.00055</td>
</tr>
<tr>
<td>40810_at</td>
<td>SWI/SNF-related, matrix-associated, subfamily c, member 1</td>
<td>SMARCC1</td>
<td>0.00061</td>
</tr>
<tr>
<td>38151_at</td>
<td>CCAAT/enhancer-binding protein (C/EBP), beta</td>
<td>CEBPB</td>
<td>3.73e–09</td>
</tr>
<tr>
<td>36330_at</td>
<td>Cysteine conjugate-beta lyase; cytoplasmic</td>
<td>CCB1</td>
<td>0.000029</td>
</tr>
<tr>
<td>37861_at</td>
<td>CD1E antigen, e polypeptide</td>
<td>CD1E</td>
<td>0.000005</td>
</tr>
<tr>
<td>38354_at</td>
<td>Early growth response 2 (Krox-20 homologue, Drosophila)</td>
<td>EGR2</td>
<td>3.92e–10</td>
</tr>
<tr>
<td>35834_at</td>
<td>Neutrophil activating protein CEBP, beta</td>
<td>CEBPB</td>
<td>3.73e–09</td>
</tr>
<tr>
<td>35836_at</td>
<td></td>
<td></td>
<td>4.40e–11</td>
</tr>
<tr>
<td>35870_at</td>
<td>Cysteine and glycine-rich protein 1</td>
<td>CSRP1</td>
<td>0.00000002</td>
</tr>
<tr>
<td>35686_at</td>
<td>Neuronal pentraxin receptor</td>
<td>NPTXR</td>
<td>0.0000056</td>
</tr>
<tr>
<td>35687_at</td>
<td>Highly similar to Homo sapiens BNPI mRNA for brain-specific Na-dependent inorganic phosphate cotransporter.</td>
<td></td>
<td>0.00000004</td>
</tr>
<tr>
<td>36567_at</td>
<td></td>
<td></td>
<td>0.000025</td>
</tr>
<tr>
<td>33956_at</td>
<td>Lymphocyte antigen 96</td>
<td>LY96</td>
<td>0.000002</td>
</tr>
<tr>
<td>37203_at</td>
<td>Carboxylesterase 1 (monocyte/macrophage serine esterase 1)</td>
<td>CES1</td>
<td>0.00017</td>
</tr>
<tr>
<td>35752_at</td>
<td>Cholesterol-24-hydroxylase</td>
<td>C24H2O7</td>
<td>0.000016</td>
</tr>
</tbody>
</table>

NOTE: Genes in bold font are common to RD-EF cells, HNFF-EF cells, and EFT cells. Genes that are inducted at different time points in RD-EF cells are shown separately in the table.
Moreover, the up-regulation of CCK (Figs. 1D and 2C, m-o), whose expression differentiates the parasympathetic neural phenotype of EFT from the sympathetic neural phenotype of neuroblastoma, clearly indicated that a specific Ewing-like parasympathetic neural phenotype was imposed in RD cell by EWS-FLI1 expression.

We also examined the effect of EWS-FLI1 expression on myogenesis, cognizant of the profound loss of morphologic evidence of myogenesis noted in Fig. 2. Consistent with the morphology, we found a striking loss of expression of key genes necessary for myogenesis, especially the myogenic transcription factors MyoD and myogenin, the myogenic intermediate filament desmin, and muscle-associated genes cholinergic receptor α and cholinergic receptor δ. Two other myogenic transcription factors, myf5 and myf6, were unaffected by EWS-FLI1 expression (Fig. 3C).

Interestingly, this expression pattern of myogenic transcription factors is reminiscent of what was seen in biphenotypic sarcomas expressing EWS-FLI1 (13). Protein level confirmation of the down-regulation of MyoD and Myogenin by Western blot is illustrated in Fig. 3D, where a nearly inverse relationship between levels of EWS-FLI1 protein and these two transcription factors was noted. Immunohistochemical staining is consistent with the microarray and Western results (Fig. 2C, d-i). Moreover, Cyclin D3, the major D-type cyclin expressed in rhabdomyosarcoma (17) and associated with muscle differentiation (28, 29), was down-regulated to an almost undetectable level after EWS-FLI1 induction, in parallel with the increase of cyclin D1 (the major D-type cyclin in EFT; Figs. 1D and 3C; Supplementary Fig. 1B, ref. 17). These data indicate that EWS-FLI1 expression induces a profound down-regulation of the muscle differentiation program.

Comparison of up-regulated genes in RD-EF system and highly expressed genes in Ewing’s family tumors revealed genes crucial in neural crest development and WNT signaling pathway. In an attempt to specify genes that were significantly up-regulated by EWS-FLI1, we first identified a list of 865 “EFT signature genes” of the 12,625 probe sets on the HU95av2 arrays, that are highly associated with primary EFT from patients (P < 0.001) and are expressed at least 2-fold higher than in the rest of the tumors. PCA analysis showed that at 24 and 36 hours, tetracycline-treated RD-EF cells were completely separated from the controls and the EWS-FLI1 protein level was stable and comparable with the original EFT cell lines. Thus, we compared the gene expression pattern of the T24 and T36 samples with the controls, including untreated and all of the ethanol-treated samples, and selected 370 genes that are highly associated with T24 and T36 (P ≤ 0.001). Of these EWS-FLI1–induced genes, 109 (30%) are also signature genes of the EFT (Table 1; Fig. 4D; Supplementary Fig. 2). Hierarchical clustering of the RD-EF samples separated these 109 genes in a temporal manner (Fig. 4A).

Two main gene expression patterns within the time course were identified: genes that were up-regulated at early time points (T6 and T12) and late time points (T18, T24, and T36). We screened the gene lists by searching the literature as well as by their Gene Ontology Annotation. Strikingly, a marked number (30%) of EWS-FLI1–up-regulated genes are important for neural crest development, such as EGR2 (Krox20), MSX1, CITED2, c-MYC, ID2, Cadherin 11, RUNX3, and Rho family members [ARHH (RhoH) and ARHGEPF1]. This is grossly disproportionate to the relative abundance of such genes by gene ontology code (P < 0.0001) and strongly suggests that a primary function of EWS-FLI1 is to invoke a form of neurogenesis. Other neural associated genes induced by EWS-FLI1 included neuronal pentraxin receptor, synaptotagmin I, SMA5, presenilin 1, as well as GABARAPL, XPNPEP1, DYSYL2, C5R1, and OLFM1.

Further inspection revealed that 20 of the 109 up-regulated genes are well-known WNT signaling components, involving both canonical and noncanonical WNT signaling pathways (Fig. 5A; ref. 30). This observation implied that WNT signaling is possibly an important mechanism whereby EWS-FLI1 invokes its biological effect, especially because WNT signaling is important in neural crest development and aberrant WNT signaling has been implicated in diverse human cancers.

Confirmatory QRT-PCR was done on selected neural genes (Fig. 4B). The data showed a high correlation (almost identical patterns) between microarray and QRT-PCR results. Because the RNA for microarray and for QRT-PCR analyses was from separate experiments, this consistency confirmed the reliability of the microarray results as well as the reproducibility of the RD-EF system. In addition, we also used siRNAs against the EWS-FLI1 breakpoint (siEFBP2, sequence from ref. 18) to inhibit EWS-FLI1 before induction. Twelve hours after transfection of siEFBP2 or a scrambled control siC8, RD-EF cells were treated by tetracycline or ethanol for 24 hours before RNA was harvested. EWS-FLI1 expression level in siEFBP2-transfected cells induced by tetracycline was only 20% of those in siC8-transfected cells induced by tetracycline. In accordance with the EWS-FLI1 level, the expression of the neural genes was also lower in tetracycline-induced siEFBP2 cells than the siC8 controls (Fig. 4C).

Correlation of EWS-FLI1 expression and the induced targets was also validated by introducing siEFBP2 into an EFT cell line TC32. EWS-FLI1 RNA level was reduced >60% in siEFBP2-transfected TC32 cells compared with siC8-transfected cells (Fig. 4D). Thus, the expression of the neural genes was also reduced. These results showed that the induction of these genes, assessed by QRT-PCR, was EWS-FLI1 dependent. The level of modulation of the neural genes by EWS-FLI1 in TC32 cells was less dramatic. This is likely due to the observed lesser inhibition of EWS-FLI1 expression in these cells. Greater inhibition of EWS-FLI1 expression is probably needed to fully abrogate its modulation of downstream genes.

Up-regulation of neural crest genes was confirmed in another cell lineage expressing EWS-FLI1. Our result suggests that EWS-FLI1 is a potent differentiation factor that blocks a preexisting commitment to myogenesis in rhabdomyosarcoma whereas imposing neural differentiation. We sought to determine whether neural differentiation is unique to the skeletal muscle cellular milieu found in RD cells, or is a generalizable effect of EWS-FLI1. The fact that these genes are also highly expressed in primary EFT precludes the possibility that this is merely a nonspecific effect after the muscle differentiation program was inhibited. However, because cell context is very important in extrapolating EWS-FLI1’s function, we have pooled our RD-EF gene expression data with comparable expression profiles derived from primary, untreated EFT, and a similar data set derived from EWS-FLI1 transfectants in a human normal foreskin fibroblast model (HNFF-EF) kindly provided by the authors (8). Using a cutoff of P < 0.001 (and expression >2-fold increased in the case of primary EFT), we have done a multiple t test analysis of each data set to identify the genes that meet these selection criteria and compared results in a Venn diagram (Fig. 5B). A final 46 genes common to all three datasets was identified (highlighted in Table 1). These genes are of special interest,
because they are clearly up-regulated by expression of EWS-FLI1, independently of cellular background, normal or malignant. Strikingly, the majority of the neural crest differentiation–related genes identified in RD-EF model were also up-regulated by EWS-FLI1 expression in this human fibroblast background. Fourteen of the 46 genes are associated with neurogenesis, and even more strikingly, of the seven genes most strongly associated with the EWS-FLI1 expression in the model systems (P < 0.000000001), the majority (5) are involved in neural differentiation, particularly neural crest development (Table 1; ref. 31). Thus, there is a strong association between neurogenesis and EWS-FLI1 expression. These data clearly indicate that neural differentiation induced by expression of EWS-FLI1 is independent of cellular background in which the gene is expressed. It excludes the possibility of a nonspecific neural up-regulation as a result of the down-regulation of myogenesis and indicates a general effect of EWS-FLI1 on differentiation.

Discussion

The traditional view of oncogenes in tumor pathogenesis is to promote proliferation, increase survival, and block the differentiation program of the target cells. However, chimeric fusion genes that result from various chromosomal translocations are different from traditional oncogenes in that they are often associated with specific tumor types (i.e., EWS-FLI1 in EFT, PAX3/7-FKHR in rhabdomyosarcoma and BCR-ABL in acute lymphoblastic leukemia, ALL). This specificity implies that the presence of the fusion gene may determine the differentiation program that the particular tumor type manifests. Transgenic mouse model studies of Philadelphia chromosome-positive (BCR-ABLp190) ALL (Ph+-ALL) indicated that the target of leukemic transformation in Ph+-ALL is normal pluripotent hematopoietic stem cells rather than committed progenitor cells and the presence of BCR-ABLp190 imposes the B-cell differentiation program of the precursor stem cells but prevents further development of the committed B-cell
precursors (32). It results in the accumulation of abnormal cells organized as a hierarchy, which failed to differentiate into functional B lymphocytes. Similarly, when PAX3-FKHR was introduced into NIH3T3 cells, cDNA microarray analysis indicated profound activation of the myogenic transcription program, including up-regulation of MyoD and myogenin (33). PAX3 transduced cells did not show this activation.

Our study showed that EWS-FLI1 has a profound effect on cell differentiation as well as proliferation. It can induce an Ewing-like neural crest phenotype in multiple cellular contexts whereas blocking the existing cell differentiation program, which strongly supports the hypothesis that this tumor-specific fusion protein acts as a cell lineage determinant, rather than a pure "oncogene." Otherwise conspicuous terminal skeletal muscle differentiation is nearly completely lost in RD cells expressing EWS-FLI1, supplanted by marked neural differentiation as documented by the appearance of neurites, dense core granules, and a number of neural genes, as noted above. Krox20, MSX1 (34), and c-MYC are all neural crest markers during development. Krox20 knockout mice have severely defective myelination of peripheral nerves (35). MSX1−/− mice have deficiencies in neural crest derivatives (36). C-MYC was shown an essential early regulator of neural crest development and knock-down of c-MYC by antisense ODN resulted in a loss of neural crest precursors in Xenopus embryos (37). Overexpression of ID2 can drive ectodermal cells into a neural crest phenotype instead of the epidermal lineage (38). CITED2 is a coactivator of another neural crest marker TFAP2. The expression of CITED2 is essential for the survival of neuroepithelial cells and disruption of CITED2 gene is embryonic lethal because of defects in the development of heart and neural tube (39–41). Cadherin 11 and Rho family GTPases are both related to delamination of neural crest cells from the neural tube (42–44). RUNX3 is an important regulator of the axonal projections of a subpopulation of dorsal root ganglion neurons (45).

Expression of neural structural genes, such as neuronal pentraxin receptor, synaptotagmin I, and MAPT, is also highly induced. CCK is a neuropeptide and is highly expressed in EFT (46). This is almost unique among tumor cells and served as evidence that EFT originated from parasympathetic progenitors. Another tumor type of neural crest sympathetic origin, neuroblastoma, does not express this gene. This was recently further corroborated by published observations that expression of EWS-FLI1 in a neuroblastoma cellular background (11) suppresses sympathetic neural differentiation, the hallmark feature of neuroblastoma cells, whereas MIC2 (CD99), c-MYC, and keratin 18 were all markedly up-regulated, similar to what we observed in this study. These data exclude the possibility of a nonspecific neural up-regulation and indicate a general effect of EWS-FLI1 on differentiation. The high expression of all these critical neural crest associated genes provided further evidence for a parasympathetic neural crest differentiation program in EFT.

Figure 5. Possible signaling pathway and gene regulation network initiated by EWS-FLI1. A, identified EWS-FLI1 target genes that are also called WNT signaling pathway components, including both canonical and noncanonical pathways. B, Venn diagram comparison of genes up-regulated in both RD-EF cells and HNFF-EF cells after EWS-FLI1 expression, as well as genes highly expressed in EFT cells revealed 46 genes common in all three groups. C, gene regulation network initiated by EWS-FLI1. Genes in red were up-regulated by EWS-FLI1. Genes in blue were down-regulated by EWS-FLI1. Genes in square boxes are called Wnt signaling targets.
Because James Ewing described Ewing's sarcoma >80 years ago, little has been known about the cell of origin of this group of tumors. Our results support the speculation that EFT probably originates from primitive multipotent progenitor cells that are capable of differentiating into neural crest derivatives. EWS-FLI1 fusion subsequently imposes a neural crest parasympathetic lineage direction to the cells but inhibits terminal differentiation. Eventually secondary genetic alterations lead to the malignancy of the cells. Interestingly, EFT members represent a continuum of different degrees of neural differentiation. Bone marrow stromal stem cells (MSC), which are classic mesodermal derivatives, have been shown multidifferentiated in addition to being multipotent and could be induced to differentiate into neurons (47). Considering that most EFT occurs in bone and soft tissue, MSC serves as one possible source of the cells susceptible of EWS-FLI1 transformation. A similar argument may be true in soft tissue, where pluripotent stem cells have also been described.

Despite several years of effort by multiple investigators, the basic mechanism whereby EWS-FLI1 expression results in the tumor entity that we recognize as EFT remains unknown. Little was revealed about how expression of a single chimeric "oncogene" can lead to such a profound genome-wide gene expression shift. This study does not permit us to distinguish direct from indirect targets of EWS-FLI1. However, by time course study and microarray analysis, we were able to infer a multitude of sequential downstream events and have identified an important but heretofore unidentified signaling pathway operative in Ewing's tumors, both in vivo and in vitro (Fig. 5B). C-MYC and ID2 were among the earliest up-regulated genes and ID2 has been reported to be a direct target of both c-MYC and ID2. Another up-regulated gene, FLI1 in our system. Its expression was also down-regulated dramatically by EWS-FLI1 induction when MYOD, CYCLIN D3, and P21 were already down-regulated. A possible mediator of MSXI up-regulation by EWS-FLI1 was CITED2, induced as early as 6 hours. It has been shown that MSXI is a potent inhibitor of its own promoter and this autosuppression could be counteracted by CBP/p300, which is the necessary partner of CITED2 for TFAP2 coactivation (51, 52).

The striking incidence of WNT signaling–associated genes identified in this analysis cannot be due to chance alone, WNT signaling has been found to be very important in neural crest development (53). WNTs could function as endogenous neural crest inducers in avian embryos, and inhibition of this pathway could block neural crest precursor formation in Xenopus (54). However, the classic WNT/β-catenin mediated signaling through TCF/LEF target genes is not likely active in EFT tumors (Supplementary Fig. 2). Can EWS-FLI1 hijack the classic WNT signaling downstream of β-catenin? Or does it activate a noncanonical WNT pathway, whose mechanism is still largely unknown? Or has a completely different, currently undescribed, pathway been used? Increased knowledge of the noncanonical WNT pathways will help to answer these questions.

Acknowledgments

Grant support: Molecular Pathology from LaMadrinas at CHLA (S. Hu-Lieskovetsky) and the Director's Challenge U01 (TJ. Triche, Ewing's tumor gene expression data for comparison).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked
in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dankin Chan and Minerva Mongeotti (EM Laboratory) for EM processing; Morgan Wu (clinical pathology laboratory) for immunohistochemical staining; Betty Schaub, Xuan Chen, and Sitara Waidyaratne (Microarray Core) for microarray processing and Dr. George Mecnamara (Imaging Core) for his expertise at microscopy.

References


EWS-FLI1 Fusion Protein Up-regulates Critical Genes in Neural Crest Development and Is Responsible for the Observed Phenotype of Ewing's Family of Tumors


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/11/4633

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2005/06/09/65.11.4633.DC1
http://cancerres.aacrjournals.org/content/suppl/2005/06/10/65.11.4633.DC2

Cited articles
This article cites 53 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/11/4633.full#ref-list-1

Citing articles
This article has been cited by 25 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/11/4633.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/65/11/4633.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.